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## Effect of Antilymphocyte Serum on the Hemopoietic Stem Cell

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Rabbit anti-mouse lymphocyte serum (ALS) was studied in respect to its effect on the hemopoietic stem cell. ALS markedly decreased bone marrow CFU cells in vitro and circulating CFU cells in vivo but slightly did bone marrow and spleen CFU cells in vivo. Lymphocytes and the hemopoietic stem cells have a common antigen.

The existence of the hemopoietic stem cell has been proved functionally but its morphological identity has not been unanimously determined. Small lymphocytes,<sup>1)</sup> pale transitional lymphocytes,<sup>2)</sup> monocytoïd cells<sup>3)</sup> and others<sup>4)</sup> have been put forward as candidates of the stem cells. Preliminary studies to contribute to this problem were performed in our laboratory by the use of stem cell proliferation after irradiation and isogeneic bone marrow transplantation, but the result obtained was not satisfactory. As a part of our further studies on this subject we studied the antigenicity of the stem cell by the use of rabbit anti-mouse lymphocyte serum (ALS) in view of the claim that the stem cell takes a form of lymphocyte. This study will also reveal the hematological side effect of ALS other than immunosuppression when ALS is used for prevention of rejection of transplanted organs.

### MATERIAL AND METHOD

The axillary and mesenteric lymph nodes from DDD mice were removed, excised with scissors and suspended in Tyrode solution. The lymphocyte suspension was filtered through 4 layers of gauze and  $2 \times 10^7$  cells were mixed with the same volume of Freund's complete adjuvant and injected into the footpads and several subcutaneous sites of normal rabbits. Four weeks later  $3$  to  $6 \times 10^7$  lymph node cells were injected intravenously into the same rabbits and these animals were bled seven days later by cutting ear veins. This was repeated 3 more times and all the sera obtained were pooled and inactivated at  $56^\circ\text{C}$  for 30 minutes. The serum then was absorbed with DDD mouse erythrocytes until its hemagglutinin titer became less than  $2^2$ , sterilized by the milipore filtration and stored at  $-20^\circ\text{C}$  until use. Its lymphoagglutinin and lymphocyte cytotoxicity titer using the trypanblue dye exclusion method was  $2^5$  and  $2^9$ , respectively. The number of hemopoietic stem cells was assayed as the number of colony forming units (CFU) in the spleen using Till and McCulloch's technique.<sup>5)</sup> Twelve to 16 month old female DDD mice supplied from The Kyoto University Animal Center was irradiated with 700-750R of  $^{60}\text{Co}$  gamma-rays followed by transplantation of

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isogenic bone marrow which were variously treated *in vitro* or *in vivo*. The source of gamma-rays used was a compact  $^{60}\text{Co}$  gamma irradiation facility of The Institute for Chemical Research of Kyoto University.<sup>6)</sup> Eight days later these mice were sacrificed, the spleens removed and CFU counted with a magnifying glass. Lyophilized guinea pig complement supplied by The Toshiba Chemical Company (lot #693) was used in the experiment.

## RESULT

### I. The effect of ALS on peripheral leucocyte and lymphocyte counts (Fig. 1).

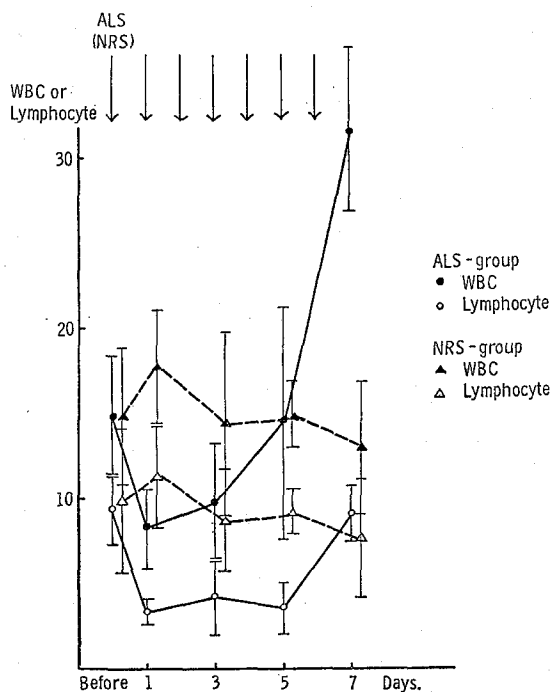


Fig. 1. WBC and Lymphocyte Count Following 7 Daily Administrations of ALS or NRS.

One quarter ml per mouse of ALS or normal rabbit serum (NRS) was injected subcutaneously daily for 7 days. In the ALS injected mice the peripheral leucocyte count dropped markedly due to the decrease of lymphocyte count. At 5 days the decreased leucocyte count went back to the preinjection level, whereas the lymphocyte count remained low indicating an increase of neutrophils. At 7 days the increase of neutrophils was more marked and the lymphocyte count was at the preinjection level. In the NRS injected mice the leucocyte as well as lymphocyte count remained essentially the same. ALS has been reported to have stronger immunogenicity than NRS and this appears to be one of the reasons why there was such a difference between the two sera.<sup>7,8)</sup>

### II. *In vitro* effect of ALS on CFU.

When mouse bone marrow cells were incubated with ALS in the presence of com-

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plement at 37°C for 30 minutes, the viability of the cells decreased markedly, whereas with NRS this was not observed (Table 1). After incubation the bone marrow cells were injected into lethally irradiated isogenic mice. A decrease of CFU count was evident as shown in A of Table 2. Increase of the bone marrow dose three times did not materially increase the number of CFU. Eight time increase of incubated transplanted bone marrow cells still did not give the same number of CFU as the control (Table 4: A versus C,  $P < 0.05$ ). In the next experiment the bone marrow cells were

Table 1. In Vitro Effect of ALS.

|   | BM cell<br>before incubation                             | Serum       | Complement | BM cell<br>after incub. | Viability<br>after incub. |
|---|--|-------------|------------|-------------------------|---------------------------|
| A | 3.5×10 <sup>6</sup> in 0.3 ml<br>of Tyrode Solu-<br>tion | (-)         | (-)        | 3.0×10 <sup>6</sup>     | 93%                       |
| B |  | (-)         | 0.1 ml     | 2.8                     | 89                        |
| C |  | NRS 0.1 ml  | (-)        | 3.0                     | 74                        |
| D |  |             | 0.1 ml     | 2.5                     | 74                        |
| E |  | ALS 0.02 ml | (-)        | 1.7+aggl. c.            | 80                        |
| F |  |             | 0.1 ml     | 3.5                     | 13                        |
| G |  | ALS 0.1 ml  | (-)        | 2.6+aggl. c.            | 78                        |
| H |  |             | 0.1 ml     | 2.2                     | 18                        |

3.5×10<sup>6</sup> Bone Marrow cells in Tyrode solution are incubated in siliconized test tubes for 30 minutes at 37°C.

Table 2. In Vitro Effect of ALS on CFU (I).

|   | Incubation              | Cells injected<br>per host | Colony counts               | Mean | S.D. |
|---|-------------------------|----------------------------|-----------------------------|------|------|
| A | ALS                     | 10 <sup>5</sup>            | 3,1,0,1,0,3,0,1,2,3,0,0     | 1.17 | 1.27 |
| B | ALS                     | 3×10 <sup>5</sup>          | 1,2,2,1,2,0,2,0,3,1,1       | 1.36 | 0.92 |
| C | NRS                     | 10 <sup>5</sup>            | 24,8,13,15,7,21,17,15,13,12 | 14.5 | 5.23 |
| D | 710R irradiated control |                            | 0,0,0,0,3,1,0,0,0,0         | 0.40 | 0.96 |

Method of incubation (37°C 30').

A & B: IBM 10<sup>7</sup> in 1 ml of Tyrode+0.033 ml ALS+0.1 ml complement.

C : IBM 10<sup>7</sup> in 1 ml of Tyrode+0.1 ml NRS+0.1 ml complement.

Table 3. In Vitro Effect of ALS on CFU (II).

|   | Incubation              | Viability<br>after inc. | Cells injected<br>per host | Colony count              | Mean | S.D. |
|---|-------------------------|-------------------------|----------------------------|---------------------------|------|------|
| A | ×100 dil. ALS           | 90.5%                   | 10 <sup>5</sup>            | 7,8,9,9,6                 | 7.8  | 1.21 |
| B | ×100 dil. Lymph-ab. ALS | 92.5                    | 10 <sup>5</sup>            | 5,5,12,6,10,6             | 7.3  | 2.97 |
| C | ×100 dil. NRS           | 87.5                    | 10 <sup>5</sup>            | 15,23,17,7,12,14,16,19,14 | 15.2 | 4.46 |
| D | Tyrode                  | 81.5                    | 10 <sup>5</sup>            | 13,10,15,15,11,12,11,5    | 11.5 | 3.59 |
| E | 710R irradiated control |                         | 0                          | 0,0,0,4,0                 | 0.8  | 1.79 |

Method of Incubation (37°C 30').

A: IBM 10<sup>7</sup> in 1 ml of Tyrode+0.1 ml of ×100 diluted ALS+0.1 ml complement.

B: IBM 10<sup>7</sup> in 1 ml of Tyrode+0.1 ml of ×100 diluted lymphocyte-absorbed ALS+0.1 ml complement.

C: IBM 10<sup>7</sup> in 1 ml of Tyrode+0.1 ml of ×100 diluted NRS+0.1 ml complement.

D: IBM 10<sup>7</sup> in 1 ml of Tyrode+ (-) +0.1 ml complement.

incubated with 100 time diluted ALS (final dilution 1000 times). After incubation, cell viability was the same as that of the cells incubated with 100 time diluted NRS. However, the CFU count was still significantly less than the control (Table 3: A versus C,  $P < 0.01$ ). This may indicate that decrease in number of CFU by ALS is not solely

Table 4. In Vitro Effect of ALS on CFU (III).

|   | Incubation                        | Viability after inc. | Cells injected per host | Colony count          | Mean | S.D. |
|---|-----------------------------------|----------------------|-------------------------|-----------------------|------|------|
| A | ALS                               | 26%                  | $8 \times 10^5$         | 8,6,9,8,3,9           | 7.2  | 2.36 |
| B | $\times 100$ ALS $\bar{s}$ Compl. | 90                   | $10^5$                  | 8,9,9,7,11,13,13,10,7 | 9.6  | 2.26 |
| C | $\times 100$ NRS $\bar{s}$ compl. | 86                   | $10^5$                  | 8,9,13,10,6,6,6,14,14 | 9.6  | 3.21 |
| D | 740R irradiated control           |                      |                         | 1,1                   | 1.0  |      |

Method of Incubation ( $37^\circ 30'$ ).

A: IBM  $10^7$  in 1 ml of Tyrode+0.1 ml of ALS+0.1 ml complement.

B: IBM  $10^7$  in 1 ml of Tyrode+0.1 ml of  $\times 100$  diluted ALS.

C: IBM  $10^7$  in 1 ml of Tyrode+0.1 ml of  $\times 100$  diluted NRS.

Table 5. In Vivo Effect of ALS.

I

|   | Irra-diation | IBM injected per mouse | ALS                          | Colony count                | Mean | S.D. |
|---|--------------|------------------------|------------------------------|-----------------------------|------|------|
| A | 750R         | (-)                    | (-)                          | 0,4,0,2,0,4,0,0,1           | 1.1  | 1.72 |
| B | 750R         | $10^5$                 | (-)                          | 15,20,16,11,5,11,18,15,5,16 | 13.2 | 5.12 |
| C | 750R         | $10^5$                 | 0.25 ml I.P. imm. before IBM | 13,8,10,12,10,9,11,5,9      | 9.8  | 2.32 |
| D | 750R         | $10^5$                 | 0.25 ml I.P. 1 day after IBM | 12,16,18,10,14,9,10,14      | 12.9 | 3.18 |

II

|   |      |        |                              |                              |      |      |
|---|------|--------|------------------------------|------------------------------|------|------|
| A | 740R | (-)    | (-)                          | 1,2,0,0,0                    | 0.7  | 0.90 |
| B | 740R | $10^5$ | (-)                          | 15,15,18,8,11,5,11,13,8      | 12.7 | 4.29 |
| C | 740R | $10^5$ | 0.25 ml I.P. imm. before IBM | 14,9,11,4,8,6,11,12,9,9,7    | 9.1  | 2.84 |
| D | 740R | $10^5$ | 0.25 ml I.P. 1 day after IBM | 18,11,15,5,8,6,4,12,14,14,16 | 11.2 | 4.77 |

III

|   | Irra-diation | IBM injected per mouse | NRS                          | Colony count     | Mean | S.D. |
|---|--------------|------------------------|------------------------------|------------------|------|------|
| A | 730R         | (-)                    | (-)                          | 0,1,0,0,0,1      | 0.3  | 0.52 |
| B | 730R         | $10^5$                 | (-)                          | 8,6,16,15,9      | 10.8 | 4.43 |
| E | 730R         | $10^5$                 | 0.25 ml I.P. imm. before IBM | 15,13,6,12,14,13 | 12.2 | 3.02 |

IV

|   | Irra-diation | IBM injected per mouse | Serum                             | Colony count                | Mean | S.D. |
|---|--------------|------------------------|-----------------------------------|-----------------------------|------|------|
| A | 750R         | $10^5$                 | ALS 0.25 ml I.P. 1 day before IBM | 9,19,18,11,10,17,13,17      | 14.3 | 3.96 |
| B | 750R         | $10^5$                 | NRS 0.25 ml I.P.                  | 18,16,13,14,18,20           | 16.5 | 2.66 |
| C | 750R         | $10^5$                 | (-)                               | 8,18,12,16,15,17,9,20,12,11 | 13.8 | 3.99 |
| D | 750R         | (-)                    | (-)                               | 0,0,0,0                     | 0    |      |

Table 6. CFU in the Bone Marrow Afer Injection(s) of ALS.

Exp. I One day after a injection of ALS.

| Treatment of marrow        |                | Nucl. cells in femoral marrow of the donors | Cells injected per host | Colony count                   | Mean | Mean per group | S.D. |
|----------------------------|----------------|---|-------------------------|--------------------------------|------|----------------|------|
| Serum                      | Dose per mouse |   |                         |                                |      |                |      |
| 1. ALS                     | 0.25 ml I.P.   | 1. $12.5 \times 10^6$                       | $10^5$                  | 13, 10, 14, 14, 13, 10, 16     | 12.9 | 12.7           | 1.73 |
|                            |                | 2. 18.5                                     | $10^5$                  | 13, 11, 12, 13, 13             | 12.4 |                |      |
| 2. ALS                     | 1 ml I.V.      | 16.2  | $10^5$                  | 4, 15, 13, 16, 12, 14, 15      | 12.6 | 12.6           | 4.07 |
| 3. NRS                     | 0.25 ml I.P.   | 1. 18.5                                     | $10^5$                  | 23, 18, 20, 19, 13, 17, 16     | 18.0 |                |      |
|                            |                | 2. 20.3                                     | $10^5$                  | 12, 19, 19, 19, 19, 17, 17, 20 | 17.8 | 17.9           | 2.47 |
| 4. Tyrode                  | 0.25 ml I.P.   | 1. 25.0                                     | $10^5$                  | 17, 16, 16, 13, 15, 15, 15     | 15.3 |                |      |
|                            |                | 2. 23.0                                     | $10^5$                  | 15, 21, 18, 14, 16, 19, 15     | 16.9 | 16.1           | 2.09 |
| 5. 700R irradiated control |                |   |                         | 0, 1, 0, 3                     | 1    |                |      |

Exp. II One day after 7 daily injections.

|        | Treatment of marrow donors |                | Nucl. cells in femoral marrow of the donors | Cells injected per host | Colony count                          | Mean | Mean per group | S.D. |
|--------|----------------------------|----------------|---|-------------------------|---------------------------------------|------|----------------|------|
|        | Serum                      | Dose per mouse |   |                         |                                       |      |                |      |
| Exp. 1 | 1. ALS                     | 0.25 ml S.C.   | 1. $13.3 \times 10^6$                       | $10^5$                  | 24, 23, 17, 18, 16, 19                | 19.5 | 18.2           | 3.15 |
|        |                            |                | 2. 15.0                                     | $10^5$                  | 20, 18, 16, 14, 15                    | 16.6 |                |      |
|        | 2. NRS                     | 0.25 ml S.C.   | 1. 11.1                                     | $10^5$                  | 15, 18, 19, 20, 12                    | 16.8 | 14.9           | 4.14 |
|        |                            |                | 2. 15.0                                     | $10^5$                  | 15, 6, 9, 11, 17, 18, 19, 14, 16      | 13.8 |                |      |
|        | 720R irradiated control    |                |   |                         | 0, 0, 0                               | 0    |                |      |
| Exp. 2 | 1. ALS                     | 0.25 ml S.C.   | 1. 10.3                                     | $10^5$                  | 11, 17, 11, 13, 19, 20, 16            | 15.3 | 13.5           | 3.30 |
|        |                            |                | 2. 12.0                                     |                         | 13, 18, 10, 11, 9, 11, 12, 14, 12, 13 | 12.3 |                |      |
|        | 2. NRS                     | 0.25 ml S.C.   | 1. 13.7                                     | $10^5$                  | 13, 14, 16, 10, 14, 14, 10, 16        | 13.4 | 13.3           | 2.19 |
|        |                            |                | 2. 12.0                                     |                         | 14, 10, 14, 15                        | 13.3 |                |      |
|        | 740R irradiated control    |                |   |                         | 0, 0, 0, 0                            | 0    |                |      |

due to its cytotoxic antibody or that the stem cells are injured more readily than the lymphocytes. Lymphocyte-absorbed-ALS whose lymphoagglutinin was decreased to  $2^2$  still had CFU decreasing ability (Table 3: B). When bone marrow cells were incubated with ALS in the absence of complement a decrease of CFU was not observed suggesting that complement is necessary for the decrease in number of CFU (Table 4).

### III. In vivo effect of ALS on CFU.

ALS was injected intraperitoneally one day and immediately before and one day after lethal irradiation and  $10^5$  isogenic bone marrow transplantation (Table 5). Splenic CFU count decreased slightly only when ALS was injected immediately before. This result indicates that the number of CFU cells decreases by an appropriate concentration of ALS while they are circulating but not readily once they enter the spleen.

### IV. CFU in the bone marrow after injection(s) of ALS.

Effect of ALS injection(s) on the number of CFU cells in the femoral marrow was studied. DDD female mice were injected with 0.25 ml of ALS intraperitoneally or with 1.0 ml of it intravenously, sacrificed 24 hours later, their femoral marrows removed and  $10^5$  cells were injected intravenously into lethally irradiated isogenic female mice. ALS injected mice had 20% less number of CFU cells in their femoral marrow than those injected with either NRS or Tyrode solution (Table 6: Exp. I). The mouse injected with 1 ml of ALS looked sick immediately after injection and the decrease in number of CFU cells in the femoral marrow could partly be due to an indirect toxic effect of ALS on the body in addition to its direct effect on lymphocytes.

Seven daily injections of ALS did not result in decrease of CFU cells in the femoral marrow (Table 6: Exp. II). As stated previously, ALS is immunogenetically stronger than NRS and at day 8 the mice had leucocytosis and almost normal lymphocyte count as shown in Fig. 1.

### V. Effect of ALS on circulating CFU. (Table 7)

Mice were injected intraperitoneally with 0.25 ml of either ALS or NRS on two successive days and on the next day after the 2nd injection the mice were bled into siliconized test tubes containing small amount of heparin. The blood thus obtained was pooled and an aliquot injected intravenously into lethally irradiated mice. The blood of mice injected with ALS contained much less number of CFU cells, the decrease being

Table 7. Effect of ALS on Circulating CFU.

|         | Treatment of<br>Blood Donor | WBC<br>(Lymphocytes)<br>of Donor Blood | Blood<br>injected per<br>host | Colony count               | Mean | S.D. |
|---------|-----------------------------|--|-------------------------------|----------------------------|------|------|
| Exp. I  | 1. ALS 0.25 ml×2            | 2100 (950)                             | 0.4 ml                        | 1,2,0,4,4,0,0,0,1,1,2      | 1.3  | 1.48 |
|         | 2. NRS 0.25 ml×2            | 4900 (3210)                            | 0.4 ml                        | 9,11,3,13,11,15,13,10,9,8  | 10.2 | 3.32 |
|         | 3. None                     | 7800 (6120)                            | 0.4 ml                        | 6,11,8,13,9,10,11,7,12,8,3 | 8.9  | 2.91 |
|         | 730R irradiated control     |  |                               | 2,0,0,0,1                  | 0.6  | 0.89 |
| Exp. II | 1. ALS 0.25 ml×2            | 2600 (1160)                            | 0.5 ml                        | 1,1,0,2,3,0,0              | 1.0  | 1.15 |
|         | 2. NRS 0.25 ml×2            | 7700 (5470)                            | 0.5 ml                        | 12,21,20,23                | 19.0 | 4.83 |

inproportionally greater than that of lymphocyte count.

### DISCUSSION

In our study the decrease of CFU cells by ALS as reported by De Meester *et al.*<sup>9)</sup> was essentially confirmed, although the results obtained were not identical in some details. The decrease was effected in the presence of complement but cytotoxic activity of ALS may not be the only factor concerned in the decrease of CFU cells because 1000 times diluted ALS and lymphocyte-absorbed ALS, both of which did not show a significant cytotoxicity in vitro, still had CFU decreasing activity as shown in Table 3. However, the problem as to whether ALS still has an anti-CFU activity after extensively absorbed with lymphocytes has not been studied. Studies in this respect are in progress.

De Meester *et al.*<sup>9)</sup> reported that the number of CFU cells decreased when bone marrow cells were incubated with ALS in the absence of complement. Their ALS dilution was only twice and it is not known what they did with cells probably aggregated by ALS of such a low dilution.

ALS decreased the number of CFU cells more readily in the peripheral blood than that in the bone marrow. Therefore, ALS appears not to penetrate into the hemopoietic organ readily. Further, the number of CFU cells went back to normal after 7 daily injections of ALS. One may not necessarily be concerned much with the bone marrow damage during daily injections of ALS after organ homotransplantation.

This study clearly shows that the lymphocyte and hemopoietic stem cell have a common antigen. However, it is not known whether only lymphocytes possess this characteristics. Studies are in progress as to the antigenicity of hepatocytes and macrophages in order to know whether they also share a common antigen with the hemopoietic stem cell.

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